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Original Article

The role of human prostate cancer specific binding peptide on the malignant phenotype of PC-3M cell

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Abstract: The objective of this study was to investigate the effect of a novel short peptide (B08: human prostate cancer specific binding peptide) on the malignant phenotype of the human prostate cancer cell line PC-3M. We developed a phage-displayed 7-mer peptide library to screen the target peptides that were specifically bound to PC-3M cells with subtractive panning from normal prostate cells and PC-3 prostate cancer cells. B08 was found to have high affinity to highly metastatic PC-3M cells. In order to explore the function of B08 on PC-3M cells, cell growth assay MTS and colony formation assay were used to determine the effect of B08 on cell proliferation. Flow cytometry (FCM) was employed to explore the apoptotic effect of B08 on PC-3M cells. The wound healing assay and transwell invasion assay were done to evaluate changes in the abilities of cell migration and invasion respectively. VEGFA at mRNA and protein levels were detected by PCR and western blotting. We found that B08 inhibited the proliferation and reduced the rate of colony formation of PC-3M cells, but did not cause a higher apoptotic rate. Additional analysis showed that B08 significantly inhibited the migration and invasion of PC-3M cells. Then we also found that B08 decreased the VEGFA mRNA and protein of PC3M cells by PCR and western blotting. In conclusion, B08 can inhibit the malignant phenotype of PC-3M, which may via down-regulating the VEGF signaling pathway *in vitro*. Thus, peptide B08 may be an effective targeted therapeutic agent for the treatment of prostate cancer.

Keywords: Prostate cancer, specific binding peptide, malignant phenotype, PC-3M

Introduction

Prostate cancer, the most common cancer in the male urinary and reproductive system [1], is difficult to be treated after the occurrence of local invasion and distal metastasis. Moreover, few targets associated with metastasis and malignancy have been identified. The five-year relative survival rate of early stage prostate cancer is >99% while that of advanced metastatic disease is only 28% [2]. However, the effects of current clinical chemotherapy are not satisfied. Therefore, novel reagents are urgently needed for the effective targeted therapeutic agent of prostate cancer.

In the early reports, the short peptide can not only specifically bond with malignant tumor cells directly but also provide drug targeted therapy of effective carrier [3]. In addition, it can improve the local concentration of drug and reduce the adverse reaction of chemotherapy medicine at the same time [3]. Several strategies have been used for the identification of tumor-associated proteins, such as serological analysis of recombinant cDNA expression libraries, ribosome display, tumor-specific antibody cloning, and phage antibody libraries [4]. In our previous study, we developed a phage-displayed 7-mer peptide library to screen the target peptides that were specifically bound to PC-3M cells with subtractive panning from normal prostate cells and PC-3 prostate cancer cells. A novel short peptide (B08: human prostate cancer specific binding peptide) was found to have high affinity to highly metastatic PC-3M cells. Peptides have the advantages of high clonal diversity, small size, rapid binding kinetics, and low immunogenicity as detection probes [5]. Therefore, studying the effect of

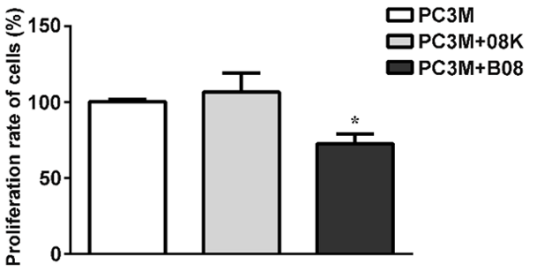

Figure 1. Effect of peptide B08 on cell proliferation of PC-3M cells. After PC3M cells were treated with peptide B08 ($4*10^{10}$ pfu) or mock-vehicle peptide 08K ($4*10^{10}$ pfu) or without any peptide, the OD value was evaluated in triplicate at 24 h. Experiments were performed at least three times. $^{*}P<0.05$.

peptide B08 on the malignant phenotype of PC-3M may have important theoretical and practical significance for the further research of specific short peptide research.

However, the effects of B08 on the malignant potential of PC-3M cells remain uncovered and the potential molecular mechanisms which B08 involves in are still unclear. In order to explore the possibility of developing B08 as a therapeutic agent and to clarify its potential pathway, we investigated the effects and the molecular mechanisms of B08 on PC-3M cells. The present study was conducted by evaluating the assessments of B08 on the proliferation, migration and invasion abilities of PC-3M cells and the involvement of the VEGF signaling *in vitro*.

Materials and methods

Cell lines and cell culture

The human prostatic cancer cells PC-3M were obtained from the key laboratory of pathobiology, Ministry of Education, Jilin University and routinely cultured in RPMI medium 1640 (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) in a humidified 37°C incubator containing 5% CO_2.

Cell growth assay

The PC-3M cells were cultured in 96-well culture plates at a cell density of 5000 cells/well, in 1640 containing 10% FBS. Following adherence overnight, the medium was replaced and the cells were incubated with different concentrations ($4*10^{10}$, $8*10^{10}$, $1.2*10^{11}$, $1.6*10^{11}$ pfu) of peptide B08 and control peptide 08K for 24 h. Viable proliferating cells were detected by the CellTiter 96®AQ_{ueous} One Solution Cell Proliferation Assay(a). Cell viability was expressed as optical density (OD), which was detected by an Automatic microplate reader (TEAN, Swiss) at a 490 nm wavelength. The inhibitory rate of cell proliferation was calculated. Seven independent experiments were performed over multiple days.

Flow cytometry (FCM) detect the apoptosis

Cell cycle analysis was performed by using propidium iodide (PI) assay. PC-3M cells were seeded at 3×10^4 cells/well into six well plates for 24 h, then treated with $4*10^{10}$ pfu peptide B08, and $4*10^{10}$ pfu peptide 08K for 24 h. Following incubation, cells were treated with PI (50 μg/mL), annexin V-FITC conjugate (5 μg/mL) and dispersed in PBS (1%, pH 7). Each analysis was performed at least thrice (n = 3) and a count of a minimum of 10,000 events was taken for each analysis. Data are represented as mean ± S.D.

Colony formation assay

Cells (1000/2 ml/well) were seeded in six well plates, and treated with $4*10^{10}$ pfu peptide B08 and 08K for 2 weeks to form colonies. The formed colonies were stained with hematoxylic, and the colonies containing >50 cells were counted under an inverted microscope.

Migration assay

The ability of cells migration ability in a monolayer culture was determined using the wound healing assay. Cells were grown to full confluence in 24-well plates and scratches were performed using a 100 μL tip. The medium was removed, and cells were washed with PBS and medium replaced by 1% FBS RPMI medium 1640 containing peptide B08, 08K ($1.2*10^{11}$). Scratch closure was analyzed under the microscope and images were captured at different time points.

Invasion assay

PC-3M cells were treated with peptide B08, 08K ($1.2*10^{11}$ pfu) for 24 h. Then cells were seeded 8×10^3 cells per well in 200 μl 1% FBS RPMI medium 1640 supplemented with B08, 08K ($1.2*10^{11}$ pfu) into the upper chamber of

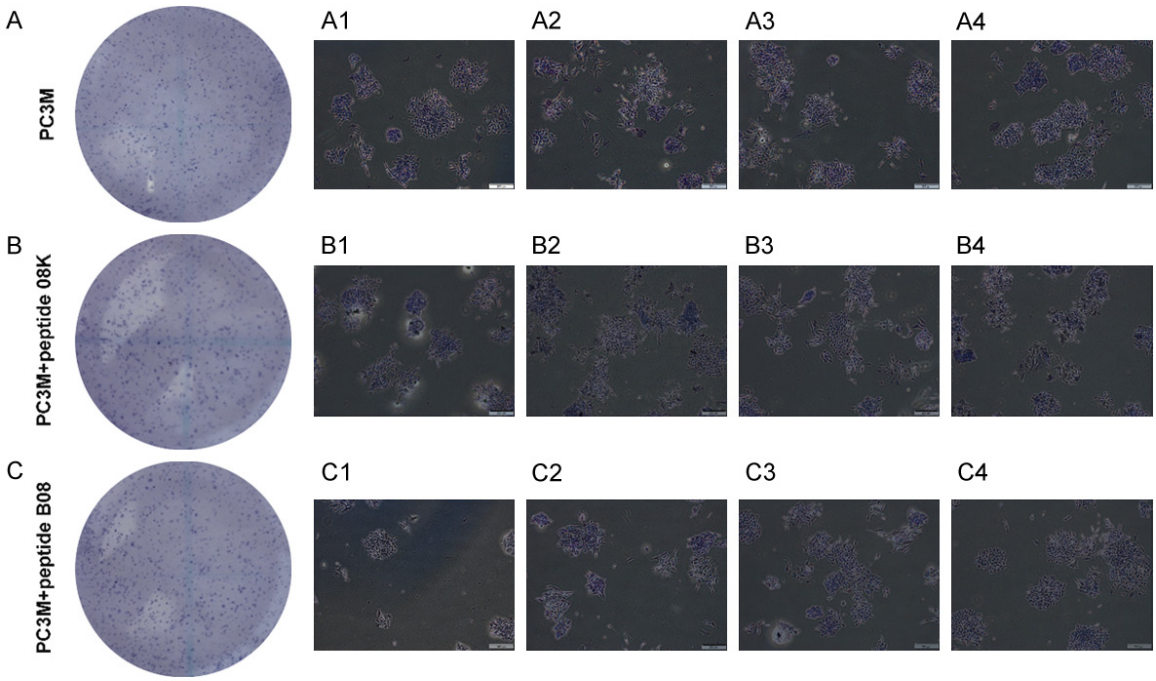

Figure 2. Effect of peptide B08 on the colony formation of the PC-3M cell line. PC3M cells were exposed to peptide B08 or 08K ($4*10^{10}$ pfu), then cells (1000/2 ml/well) were seeded in six-well plates. After 2 weeks, cell colonies were stained with hematoxylin and the colonies containing >50 cells were counted under an inverted microscope (magnification×100). Representative images of colony formation of PC3M (A, A1-A4), PC3M+08K (B, B1-B4), and PC3M+B08 (C, C1-C4). D: The histogram of number of colony formation of PC3M after treatments above. Experiments were performed at least three times. *P<0.05.

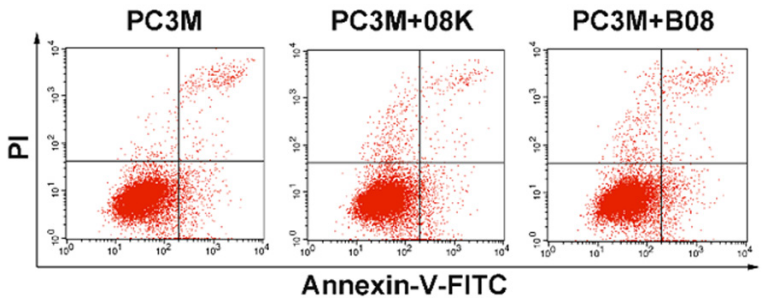

Figure 3. Effect of peptide B08 on PC-3M cells apoptosis. Annexin V-FITC/PI assay was used to analyze the percentages of apoptotic PC-3M cells, PC-3M cells treated with peptide 08K, PC-3M cells treated with peptide B08. The apoptotic rates were 6.11%, 5.31%, 6.49%, respectively.

the transwell in 24-well plates (membrane pore size, 8 μm; Corning Incorporated; Corning, NY, USA) with Matrigel (BD Pharmingen), and 500 μl 15% FBS RPMI medium 1640 was added into the lower chamber. After 24 h, the bottoms of the inserts were fixed in paraformaldehyde solution for 10 min and stained with 0.1% crystal violet staining solution. The cells invading into the bottom-lower surfaces of inserts were measured by using an inverted phase contrast microscope.

Protein extraction and western blotting

Proteins were extracted from the cells by washing them twice in ice-cold PBS and subsequently lysing them by using RIPA buffer containing 1% PMSF. Protein concentration was quantified with the BCA Protein Kit (Beyotime) and equal amounts

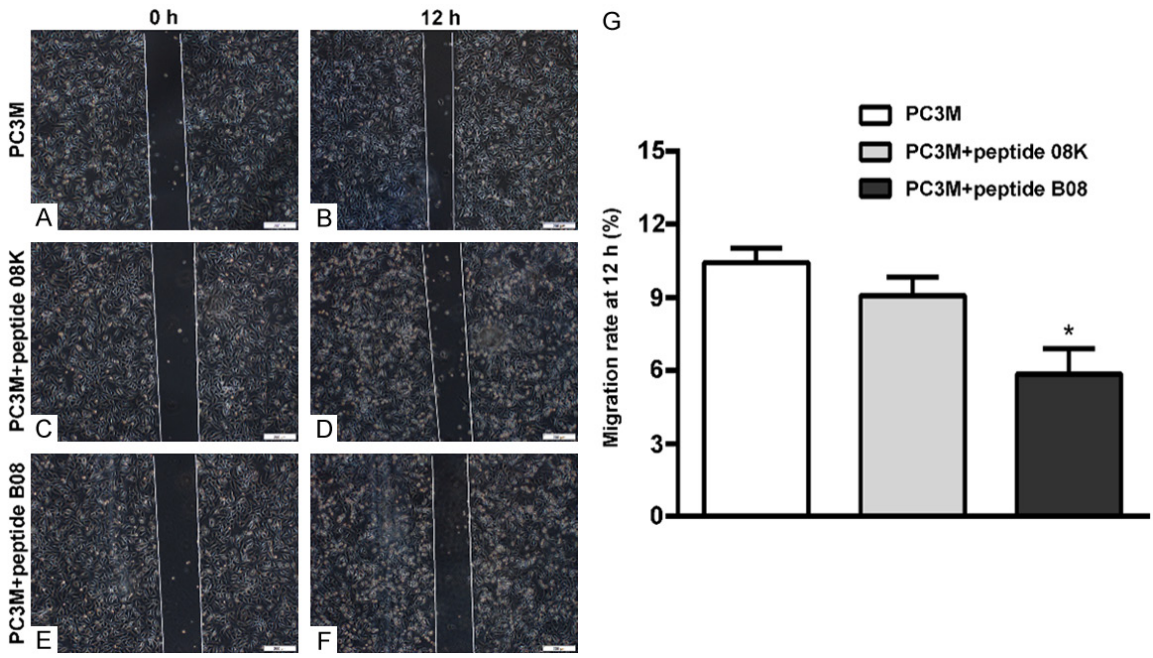

Figure 4. Effect of peptide B08 on the migration of PC-3M cells. Migrated cells were photographed at 0 h and 12 h. A and B: Representative images of migrated cells treated with no any peptide. C and D: Representative images of migrated cells treated with peptide 08K. E and F: Representative images of migrated cells treated with peptide B08. G: The histogram of migrated rate at 12 h of PC3M after treatments above. Experiments were performed at least three times. (magnification×100). $^{*}P<0.05$.

of proteins were resolved onto a 12% SDS-PAGE, transferred a to a PVDF membrane (Millpore) and probed with primary antibody anti-VEGFA, β-actin. Membranes were then incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000) and detection was performed with enhanced chemiluminescence (ECL Kit). Protein levels were normalized to β-actin.

RNA extraction, standardization of the one-step duplex RT-PCR

Total RNA was extracted from PC-3M cells using Trizol reagent according to the manufacturer's instructions (Invitrogen). The StarScript II One-step RT-PCR Kit (GenStar) carried out in 20 μL reaction volume comprising of 1 μg of RNA, 0.4 μM of forward and reverse primers of GAPDH and VEGFA, 4 μl 5X reaction buffer, 1.5 μl One-step RT/Taq Mix, add DEPC-ddH_2O to 20 μl. The reaction condition for the thermal cycles were 42°C for 30 min, 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s respectively and final extension at 72°C for 7 min. After the PCR, the products were electrophoresed on 2% agarose (Sigma-Aldrich, St. Louis, MO, USA) gel stained with ethidium bromide and visualized under gel documentation system (Tanon). The specific primers used were listed as follows: GAPDH forward: TGTTGCCATCAATGACCCCTT, reverse: CTCCACGACGTACTCAGCG; VEGFA forward: GGTGGGGTCATGTGTGTGG, reverse: AGGTCTTGTTCGCTGCCTGA.

Statistical analysis

Data are expressed as the means ± S.D. An unpaired two-tailed t test analysis was used to analyze the data from different groups. A value of $P<0.05$ was considered to indicate a statistically significant difference. All analyses were performed with SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

Results

Effects of peptide B08 on PC-3M cells proliferation in vitro

The effect of peptide B08 on the growth of the PC-3M cells was investigated using MTS and colony formation assays. The results showed that the proliferation of PC-3M cells was inhibited by peptide B08 (**Figure 1**). The colony formation rate of cells treated with peptide B08

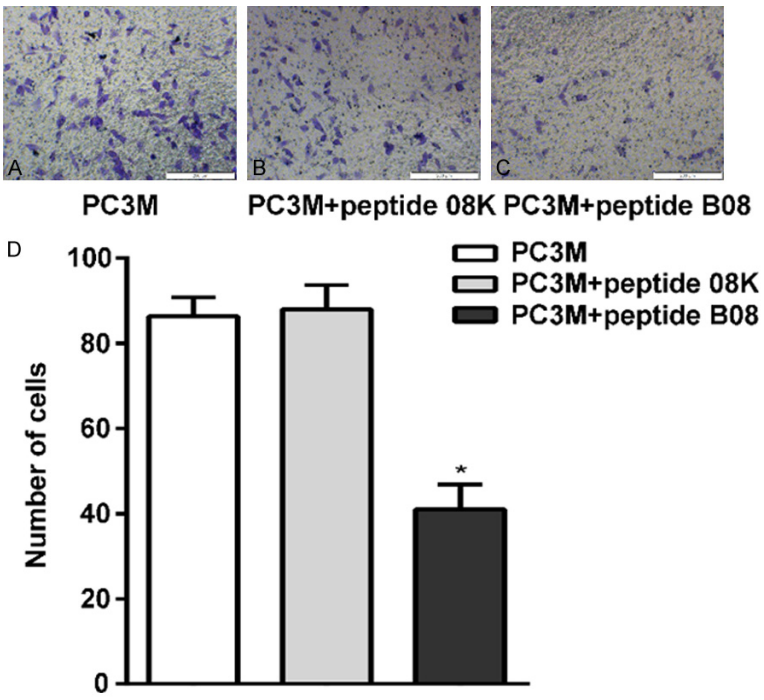

Figure 5. Effect of peptide B08 on the invasion of PC-3M cells. Invasion assay was performed after 24 h incubation. A: Representative images of invasive cells treated with no any peptide. B: Representative images of invasive cells treated with peptide 08K. C: Representative images of invasive cells treated with peptide B08. D: The histogram of number of invasive cells after treatments above. Experiments were performed at least three times. (magnification×100). $^{*}P<0.05$.

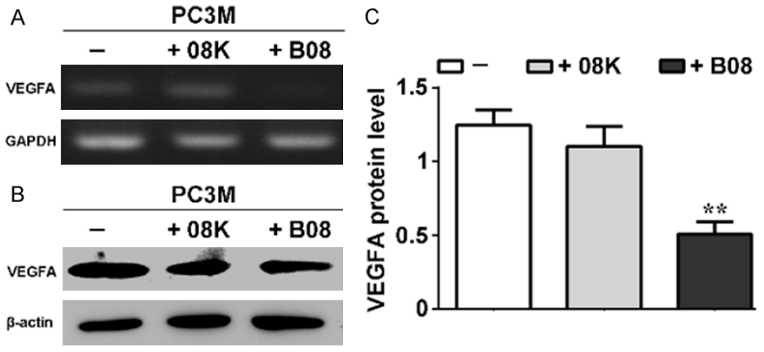

Figure 6. Effect of peptide B08 on the level of VEGFA protein. A: VEGFA and GAPDH mRNA were determined by PCR in PC-3M, PC-3M with peptide 08K and PC-3M with peptide B08. B: The cell lysates were immune blotted with anti-VEGFA and anti-β-actin antibodies respectively. C: Protein levels of VEGFA were represented as relative expression (mean ± S.E.M.) standardized by endogenous expression of β-actin. Experiments were performed at least three times. $^{**}P<0.01$.

was obviously lower than the rate of the cells treated by peptide 08K (human prostate cancer specific binding peptide 08K) or not treated by any peptide (**Figure 2A-D**). These data indicate that peptide B08 inhibits PC-3M cell proliferation *in vitro.*

Peptide B08 had no significant effect on the apoptotic cell death

FCM analysis was used to investigate the effect of peptide B08 on the induction of apoptosis of PC-3M cells *in vitro*. Peptide B08 was added to the PC-3M cells in the exponential growth phase for 24 h, and cell samples were obtained and fixed for FCM analysis. The results revealed that peptide B08 had no significant effect on the apoptotic cell death (**Figure 3**).

The invasion and migration of PC-3M were inhibited by peptide B08 in vitro

To examine the effect of peptide B08 on PC-3M cells migration and invasion, the migration and invasion capabilities were assessed by the wound healing and Transwell invasion assays. As shown in (**Figure 4A-G**), the migration rate of cells treated with peptide B08 was 41.00 ± 5.86% in the PC-3M cells compared with 88.00 ± 5.69% in the cells treated with peptide 08K and 86.33 ± 4.49% in the cells not treated with peptide. In the Transwell invasion assay (**Figure 5A-D**), the invasion of the cells treated with peptide B08 was significantly inhibited compared with that treated with peptide 08K or untreated peptide cells ($P<0.05$). This suggested that peptide B08 suppressed PC-3M cells migration and invasion *in vitro*.

Peptide B08 suppressed the activity of the VEGF signaling

To investigate the effects of peptide B08 on the activity of the VEGF (vascular endothelial growth factor) signaling, the protein expression

levels of VEGF were detected. The results showed that the protein expression levels of VEGFA were significantly decreased in the cells treated with peptide B08 when compared with these levels in the untreated cells (**Figure 6B**, **6C**). The PCR showed that the mRNA levels of VEGFA were significantly decreased in the cells treated with peptide B08 when compared with these levels in the untreated cells (**Figure 6A**). It suggested that peptide B08 suppressed the activity of VEGFA in PC-3M cells *in vitro*.

Discussion

Metastasis of prostate cancer is a complex and multistep process. It is not only involved in the biological behavior of tumor cells, but also closely related to angiogenesis of tumorous stroma and the degradation of extracellular matrix [6]. Metastatic castration-resistant prostate cancer (mCRPC) is the lethal and incurable stage of PCa [2]. Currently, chemotherapy is one of the mainstay of treatment for mCRPC. However, conventional chemotherapies have been used with limited efficacy and significant toxicity [7]. Therefore, any effort on discovering a new molecular that target-direct to PCa may contribute to the treatment of PCa.

In the process of current drug treatment of tumor, chemotherapy drugs spread not only into the tumors, but also into the healthy tissue and organs, which greatly limits the effect of drugs in the treatment of tumor [8-10]. Senthilkumar K et al reported that using biological molecules which is specific to tumor can effectively solve the problem [11]. Traditional target therapy is guided by monoclonal antibody carrier, but there are still some defects [12]. Nevertheless using short peptide to treat cancer has its unique advantages. Peptide receptors are more likely to present original conformation and the types of receptor are not necessary to be known in advance. The short peptide can specifically bond malignant tumor cells directly, provide effective carrier to the targeted drug, improve the local concentration of drug and reduce the adverse reaction of chemotherapy medicine at the same time [3]. Phage display random peptide library technology [13, 14] can be applied for obtaining the specific binding peptides of target cells.

In the preceding work of our project group, we obtained specific binding peptide B08 through phage display random peptide library technology. Therefore, studying the effects of peptide B08 on the malignant phenotype of PC-3M have important theoretical significance and practical significance for the further research of specific short peptide research.

In order to examine the effects of peptide B08 on PC-3M cell proliferation, migration and invasion, the cell proliferation was evaluated with MTS and colony formation assays, and cell migration and invasion were assessed by wound healing and Transwell invasion assays. We found that peptide inhibited the proliferation of PC-3M cell and the rate of colony formation of peptide B08-treated cells was significantly lower than that in cells treated with peptide 08K or not treated with peptide. But peptide B08 had no significant effect on the PC-3M apoptosis, the specific mechanism remains to be further discussed. In the wound healing and Transwell invasion assays, the results revealed that the migratory and invasive capabilities were inhibited by peptide B08. These results indicate that the malignant phenotype of PC-3M cells may be inhibited by peptide B08 *in vitro*. Peptide B08 may be an effectively agent for chemotherapy in the treatment of prostate cancer. However, further studies are necessary to unveil the potential molecular mechanisms of the inhibition of the malignant phenotype of prostate cancer by peptide B08.

In the early reports, short peptides can specifically inhibit MMP-2 and MMP-9 in ovarian carcinoma and fibrosarcoma [15]. In murine LCC cells, a phage-displayed tumor-homing peptide can block angiogenesis by specifically binding to MMP-2-Processed collagen IV [16]. Our findings suggested that peptide B08 altered the cell malignant phenotype of PC-3M cell via downregulation of the activity of the VEGFA *in vitro*. But further study is needed for the mechanisms. Recently, studies have shown that target metabolic pathways such as VEGF/Angiogenesis may represent a promising therapeutic strategy in cancer therapy. Angiogenesis is critical for tumor growth [17]. Liu C et al reported that there was also accumulating evidence that genetic changes were found in tumors to increase VEGF expression, an effect that is additive with hypoxia [18]. Hypoxia-inducible factor 1 (HIF-1) has been a close relation with prostate cancer [19]. Therefore, the peptide

B08 could down regulate the activity of the VEGF in PC-3M *in vitro*. Further study is needed for the mechanism to confirm whether B08 regulates the activity of the VEGF by influencing HIF-1α.

In conclusion, our findings suggested that peptide B08 could alter the cell malignant phenotype of PC-3M cell via downregulation of the activity of the VEGFA *in vitro* and may be an effective chemotherapeutic agent for prostate cancer. On account of the tumor microenvironment playing an important role in tumor progression, invasion and cell migration [20], further experiments *in vivo* are necessary to ascertain whether peptide B08 represents a new chemotherapeutic agent for the treatment of prostate cancer.

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Disclosure of conflict of interest

None.

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